American Journal of Medical Genetics 64:329–333 (1996)

Tissue-Specific Methylation Differences and Cognitive Function in Fragile X Premutation Females

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Tissue-specific variation in $(CGG)_n$ repeat size and methylation status of the FMR1 gene was investigated in 17 female premutation carriers. Minor variation in premutation repeat size among leukocyte, lymphoblast, and fibroblast tissues was noted in some subjects. One subject exhibited a premutation size allele of $(CGG)_{64}$ in leukocyte and fibroblast tissues by polymerase chain reaction analysis but a normal-size allele of $(CGG)_{46}$ in lymphoblast cells, suggesting low-level mosaicism in blood and clonality of the lymphoblast cell line. Six subjects exhibited differences in methylation pattern between leukocytes and lymphoblasts but not between leukocytes and fibroblasts, whereas 2 subjects showed large differences in methylation pattern between leukocytes and fibroblasts. Cognitive function was studied in 14 subjects using the Wechsler Adult Intelligence Scale—Revised. Mean Verbal and Performance IQs were well within the average range as was the mean Full Scale IQ; nevertheless, a trend toward lower Performance IQ compared with Verbal IQ was observed. No significant correlation was apparent between Full Scale IQ and $(CGG)_n$ repeat size; however, a significant positive correlation was observed between Full Scale IQ and the proportion of the active X carrying the normal FMR1 allele in fibroblasts but not in leukocytes or lymphoblasts. © 1996 Wiley-Liss, Inc.

KEY WORDS: fragile X syndrome, FMR1, premutation, methylation, IQ

INTRODUCTION

Fragile X syndrome, or Fra(X), is caused by an unstable mutation in a trinucleotide $[(CGG)_n]$ repeat region located in the 5′ untranslated region of the FMR1 gene [Fu et al., 1991]. The trinucleotide repeat expansion is the site of FRAXA, the cytogenetically visible folate-sensitive fragile site at Xq27.3 [Lubs, 1969; Sutherland, 1977]. Normal alleles have 6–54 repeats, premutations have 52–200 repeats, and full mutations have more than 200 repeats [Fu et al., 1991]. The Fra(X) phenotype is not thought to be caused by trinucleotide expansion per se but rather by the subsequent methylation of the expanded $(CGG)_n$ region and the adjacent CpG island, resulting in a lack of expression of the FMR1 gene [Pieretti et al., 1991].

Variability in phenotypic expression is seen in affected males and females with full mutations. Mosaicism for $(CGG)_n$ repeat size and for methylation status, which could affect phenotype, is common [Oberlé et al., 1991] and tissue-specific differences with respect to $(CGG)_n$ size have been reported in both affected males and females [Wöhrle et al., 1992; Taylor et al., 1994]. In females with a full mutation, about half are mentally retarded [Rousseau et al., 1991], and, although an association was observed between the proportion of active X chromosome carrying the normal FMR1 allele and IQ in leukocytes by Abrams et al. [1994], no significant relation was observed by Taylor et al. [1994]. Thus, mosaicism may complicate the predictive value of testing for Fra(X) by using simple DNA analysis of a single tissue, usually peripheral blood.

Female premutation carriers do not exhibit any overt negative effects of the premutation [Reiss et al., 1993; Taylor et al., 1994; Thompson et al., 1994]; nevertheless, a tendency toward emotional and neuropsychological deficits [Sobesky et al., 1994] and subtle physical manifestations [Hull and Hagerman, 1993] have been reported. The purpose of the present study was to ex-

Received for publication September 19, 1995; revision received January 17, 1996.

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amine the incidence of tissue-specific mosaicism by comparing $(CGG)_n$ repeat size, methylation patterns, and cytogenetic fragile site expression in leukocyte, lymphoblastoid, and fibroblast tissues from premutation carrier females. In addition, cognitive ability was studied in a subgroup of the same subjects by using the Wechsler Adult Intelligence Scale—Revised (WAIS-R) [Wechsler, 1981].

MATERIALS AND METHODS

Subjects

Seventeen female premutation carriers, 30–65 years old, were recruited from known Fra(X) families to participate in the study. Participants F1 and F3 are the mothers of F9 and F11, respectively. F5 and F8 are sisters. Participants F2, F4, F6, F7, and F13 are related as follows: F6 and F7 are sisters, F6 is the mother of F13, and F6 and F7 are the maternal aunts of F2 and F4. Six remaining participants (F10, F12, F14–F17) are not related to the other participants. Blood was drawn for molecular and cytogenetic analyses and to establish a lymphoblastoid cell line. A punch biopsy was taken for a fibroblast culture, and fibroblast cultures were examined at an early passage (1–4 doublings).

Cytogenetic Analysis

Fragile site expression in lymphocyte cultures was induced by growth in medium 199 supplemented with 5% fetal calf serum [Sutherland, 1977] and by the addition of 10^{-7} M FUdR during the final 24 hr of culture [Glover, 1981]. Lymphoblast cultures were grown in duplicate to a density of 5×10^5 cells/mL [Krawczun et al., 1986] and 10^{-7} M FUdR [Jacobs et al., 1982] or 600 mg/L thymidine, respectively, was added during the last 24 hr. Induction was achieved in fibroblast cultures by the addition of 600 mg/L thymidine for the last 24 hr of culture [Sutherland and Baker, 1986]. Fragile site expression was scored in cells from the lymphocyte, lymphoblast, and fibroblast cultures.

Molecular Analysis

DNA was extracted from leukocytes, lymphoblasts, and fibroblasts by using established protocols [Maniatis et al., 1989]. The length of the FMR1 $(CGG)_n$ repeat was determined as described by Fu et al. [1991] with the following modifications. Primer c was 5′ end labeled with 5-carboxy-fluorescein (FAM); 300 ng of template DNA was used, and polymerase chain reaction (PCR) products were separated on a 6% denaturing polyacrylamide gel (Novex) with an ABI 373a automated sequencer in the presence of an internal lane standard (Genescan-1000; ABI). Fragment sizes were determined with Genescan 672 software.

For Southern blot analysis, 3 μg of genomic DNA was digested with *Eco*RI and *Bss*HII for at least 5 hr at 37°C and separated on a 20-cm 0.6% agarose/0.5X TBE (Bio-Rad) gel. Following overnight transfer to Hybond N+ membrane, the blot was probed with the 1-kb *Pst*I fragment of probe pE5.1 labeled with ^{32}P-dCTP [Fu et al., 1991]. Overnight and 3-day X-ray exposures were examined to ensure that no low-level mosaicism was missed. FMR1 band sizes were estimated from blots by comparison with size standards run in the first lane of each gel. The areas of the unmethylated and methylated bands were determined by phosphorimaging. Blots were exposed to a phosphor screen overnight and scanned in a phosphorimager (Molecular Dynamics, Inc). Band area was calculated with ImageQuant software (version 3.3), and the proportion of the active X chromosome that was normal was calculated as the area of normal unmethylated band divided by the sum of the area of normal unmethylated band and the area of mutant unmethylated band.

Cognitive Tests

Fourteen subjects were examined with the WAIS-R, which measures verbal and nonverbal cognitive functioning. The WAIS-R consists of 11 subtests: information, digit span, vocabulary, arithmetic, comprehension, similarities, picture completion, picture arrangement, block design, object assembly, and digit symbol. The subject's performance is represented in terms of scaled scores from 1 to 19 points, with a mean of 10 and a standard deviation of 3. IQ scores are obtained by transforming the sum of scaled scores in the Verbal domain (VIQ; first 6 subtests), the Performance domain (PIQ; last 5 subtests), and the Full Scale (FSIQ) to a new scaled score with a mean of 100 and a standard deviation of 15. Three factor scores that capture the main themes or dimensions of the underlying performance on the WAIS-R and are represented by deviation IQs were derived according to guidelines described by Sattler [1992]. These 3 factors and the subtests representing each of them are verbal comprehension (VC): information, vocabulary, comprehension, and similarities; perceptual organization (PO): picture completion, block design, and object assembly; and freedom from distractibility (FD): digit span and arithmetic.

RESULTS

Cytogenetic Analyses

In lymphocytes, 16/17 subjects did not exhibit fragile X expression in 150 cells, and 1 subject (F6) had fragile X expression in 2/150 cells (1.3% expression). In lymphoblasts, no subject showed fragile X expression in 150 cells. In fibroblasts, 16/17 subjects exhibited no fragile X expression in 75 cells, and 1 subject (F13) expressed fragile X in 1/75 cells (1.3% expression). Cytogenetic results are summarized in Table I.

Molecular Analyses

PCR and Southern blot analysis of the $(CGG)_n$ repeat region revealed that all 17 subjects had premutation size alleles (60 to ~135 repeats) in their leukocytes and fibroblasts and 16/17 subjects had premutation size alleles in their lymphoblasts (Table I). In general, repeat size showed only minor variation among tissues. Three subjects (F3, F8, and F13) had differences of 10 or more repeats among tissues, but all were within the premutation range. One subject (F9) was found to have premutation size alleles of $(CGG)_{64}$ in her leukocytes and fibroblasts but a normal size allele of $(CGG)_{46}$ in her lymphoblasts. Microsatellite analysis confirmed that all 3 samples were from the same individual (data not

TABLE I. Summary of Cytogenetic and Molecular Data for 17 Premutation Fragile X Females

Subject	Age	Full Scale IQ	Leukocytes DNA[a]	Leukocytes FraX[b] (%)	Lymphoblasts DNA	Lymphoblasts FraX (%)	Fibroblasts DNA	Fibroblasts FraX (%)
F1	64.2	113	29, 60	0	29, 60	0	29, 57	0
F2	36.9	104	43, 71	0	43, 71	0	43, 72	0
F3	57.7	91	30, 72	0	30, 61	0	30, 71	0
F4	39.5	119	41, 76	0	43, 76	0	43, 76	0
F5	38.6	93	30, 84	0	31, 92	0	31, 92	0
F6	61.8	133	30, 85	1.3	30, 85	0	30, ~85[c]	0
F7	64.4	95	31, 81	0	31, 83	0	31, 83	0
F8	41.2	101	31, 82	0	31, 68	0	32, ~80[c]	0
F9	41.2	112	33, 64	0	33, 46	0	33, 64	0
F10	35.9	95	21, 88	0	21, 90	0	21, ~90[c]	0
F11	31.7	96	36, 92	0	37, 101	0	36, ~100[c]	0
F12	57.3	96	21, 104	0	21, ~100[c]	0	21, ~100[c]	0
F13	38.9	124	30, 105	0	30, 87	0	30, ~90[c]	1.3
F14	34.0	109	23, 81	0	23, 82	0	23, ~80[c]	0
F15	45.4	nd[d]	31, 106	0	31, ~100[c]	0	31, ~100[c]	0
F16	31.5	nd	31, ~135[c]	0	31, ~135[c]	0	31, ~135[c]	0
F17	31.9	nd	30, 79	0	30, 79	0	30, 79	0

[a] $(CGG)_n$ repeat sizes.
[b] Percentage of cells expressing a fragile site at Xq27.3.
[c] Premutation size estimated from Southern blot analysis.
[d] nd = IQ testing not done.

shown). No significant correlation was observed between premutation repeat size and FSIQ ($r = -0.15$, $P = 0.330$).

The area of the unmethylated band of the normal allele divided by the total area of the two unmethylated bands was determined by densitometry, and the proportion of the active X (unmethylated) that carried the normal allele was calculated. The proportion of the active X with the normal allele showed some variability from tissue to tissue and ranged from 0% to 100% among subjects (Fig. 1). The lymphoblasts were most variable, with large differences observed between leukocytes and lymphoblasts in 6 subjects. Two subjects (F12 and F15) showed large differences between leukocytes and fibroblasts (74.0 vs. 40.2% for F12 and 73.3 vs. 40.8% for F15). No correlation was found between methylation status in leukocytes and FSIQ or in lymphoblasts and FSIQ ($r = -0.12$, $P = 0.69$ and $r = 0.30$, $P = 0.32$, respectively), but a significant correlation was observed between methylation status in fibroblasts and FSIQ ($r = 0.55$, $P = 0.05$).

Cognitive Tests

Table II summarizes means and standard deviations for the WAIS-R VIQ, PIQ, and FSIQ, the VC, PO, and FD factor-deviation IQs, and the individual subtest scale scores. The mean VIQ and PIQ fell in the clinically average range, although PIQ tended to be lower. The mean VC, PO, and FD factor-deviation IQs similarly fell within the high average range, again with PO tending to be the lowest of these 3 quotients.

Individual subject's WAIS-R profiles of strengths and weaknesses were examined to determine whether there were any consistent cognitive patterns. Because many possible test score comparisons on a relatively small number of subjects may produce unreliable interpretations, analyses focused on differences between VIQ and PIQ and among factor-deviation IQs, which were considered to represent meaningful summaries of the subjects' performances. Using criteria described by Sattler [1992] to examine these differences reliably, a significance level of $\alpha = 0.01$ was used. Twenty-one percent of the subjects had a significant VIQ–PIQ difference, with PIQ being lower in each case. The average

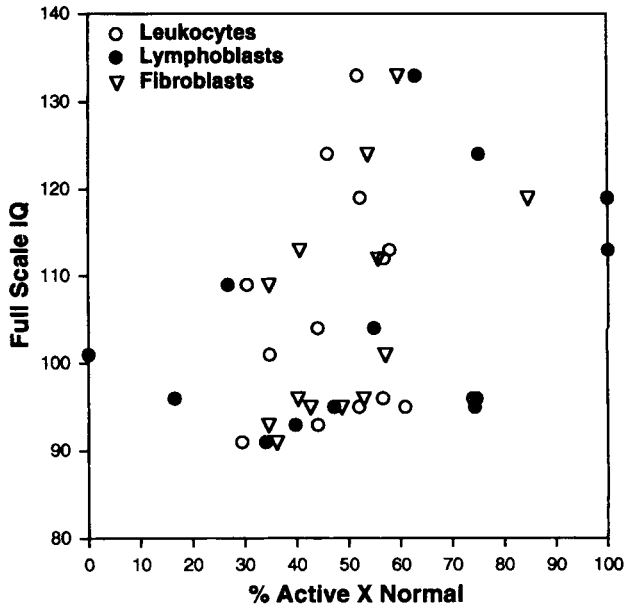

Fig. 1. FSIQ versus percentage of the active X carrying the normal allele in leukocytes, lymphoblasts, and fibroblasts (active normal X/(active normal X + active mutant X)). A significant positive correlation was observed between fibroblasts and FSIQ ($r = 0.55$, $P = 0.05$).

TABLE II. WAIS-R IQ[a] and Verbal and Performance Scaled Scores for Premutation Fragile X Females

Variables[b]	Mean (SD); n = 14
WAIS-R IQs	
VIQ	107.1 (13.7)
PIQ	103.0 (11.2)
FSIQ	105.8 (13.0)
Factor-deviation IQs	
VC	105.3 (13.1)
FD	108.8 (12.8)
PO	102.0 (12.2)
Verbal subtests	
Information	9.0 (2.4)
Digit span	10.9 (3.3)
Vocabulary	11.3 (3.1)
Arithmetic	11.7 (2.5)
Comprehension	11.0 (2.8)
Similarities	11.0 (2.3)
Performance subtests	
Picture completion	8.7 (2.3)
Picture arrangement	9.7 (2.7)
Block design	9.9 (2.7)
Object assembly	8.4 (2.2)
Digit symbol	9.8 (2.9)

[a] Wechsler Adult Intelligence Scale—Revised [Wechsler, 1981].
[b] VIQ, Verbal domain; PIQ, Performance domain; FSIQ, Full Scale; VC, verbal comprehension; FD, freedom from distractibility; PO, perceptual organization.

size of the discrepancy observed for these subjects is a difference that is estimated to occur in the WAIS-R standardization sample only 12.2% of the time [Sattler, 1992]. Comparisons of factor-deviation IQs, which are regarded as measures purer than VIQ and PIQ of the primary dimensions in the WAIS-R [Sattler, 1992], indicated that 57% of subjects showed significant discrepancies for one or more of their scores. Of these, 75% had PO scores that were significantly lower than VC, FD or both, whereas FD was significantly higher than either or both of the other factors.

DISCUSSION

These data indicate that size mosaicism of the $(CGG)_n$ repeat number among different tissues does exist in carrier females but that the differences are slight and usually would not affect the diagnosis of carrier or noncarrier. However, 1 carrier (F9) had a premutation in both leukocytes and fibroblasts but a normal size allele in lymphoblasts. Because this subject and her mother had a small premutation size allele of $(CGG)_{57-64}$, the normal size allele seen in the lymphoblast culture is likely derived from a revertant cell that was cloned to form the line. These findings emphasize the clonality of cultured cell lines and suggest that such cell lines should not be used diagnostically. Another argument against the use of lymphoblast lines is the finding that 6/17 (35.3%) of subjects showed large methylation differences between leukocytes and lymphoblasts versus 2/17 (11.8%) of subjects with methylation differences between leukocytes and fibroblasts. FMR1 expression is not thought to be affected by premutations; however, this conclusion was drawn from studies on lymphoblast cell lines [Feng et al., 1995]. The findings of the present study suggest that the use of lymphoblast cell lines to study FMRP expression may not reflect in vivo conditions and that results from such cell lines should be interpreted with care.

Cytogenetic expression of a fragile site at Xq27-q28 was observed in 1.3% of cells in 1 of 3 tissues examined for subjects F6 and F13. Although this may represent low-level FRAXA expression of the premutation allele or undetected mosaicism for a full mutation, the fragile site expression may be unrelated to carrier status because fragile sites were also occasionally observed in noncarrier female members of Fra(X) families (data not shown). Cytogenetic expression of FRAXA is generally thought to be correlated with repeat number and uncommon among premutation carriers [Yu et al., 1992].

A significant positive correlation was observed between the proportion of the active X carrying the normal allele in fibroblasts and FSIQ. In leukocytes and lymphoblasts, no significant correlation was observed, in agreement with previous findings [Taylor et al., 1994]. Although additional studies are required to determine whether the positive correlation observed in fibroblasts can be confirmed, fibroblasts may prove to be a more appropriate tissue than leukocytes or lymphoblasts for studying the correlations between repeat length and/or methylation status and intellectual functioning.

VIQ, PIQ, and FSIQ all fell in the clinically average range, suggesting that there was no significant effect of premutation carrier status on overall intelligence, in agreement with previous reports [Reiss et al., 1993; Taylor et al., 1994; Thompson et al., 1994]. However, this conclusion must be regarded with caution because of the small number of subjects and the fact that this study was not adjusted for the intelligence level of the subjects' parents. Profile analysis of individual subjects, which is also limited by the small sample size, was suggestive of a trend toward relative weaknesses in aspects of perceptual organizational/performance abilities. A similar trend has been noted in previous studies that presumably included both premutation and full-mutation carriers [Kemper et al., 1986; Miezejeski et al., 1986], suggesting that the possibility that premutation carriers may not be asymptomatic in this regard should be investigated in a larger group of subjects. In the present study, a relative strength in FD was noted.

In summary, the findings of this study suggest that size and methylation differences do exist among different tissues from premutation carriers and that sufficient differences are observed between leukocytes and cultured cell lines, such that care should be taken when extrapolating data obtained from studying cell lines to individuals from whom the cell lines were derived. A significant positive correlation observed between the proportion of the active X carrying the normal allele in fibroblasts and FSIQ but not in leukocytes or lymphoblasts and FSIQ suggests that mutational analyses in fibroblasts may have greater validity. Finally, although overall intelligence does not appear to be affected by premutation carrier status, specific strengths and deficiencies are evident. The data indicate that a multicenter collaborative study is required to collect data on a sufficient number of subjects to determine if the trends observed in the present study are significant.

ACKNOWLEDGMENTS

We thank X. Wu and M. Eliou for performing the cytogenetic analyses and Dr. David Nelson, Baylor College of Medicine, for providing the pE5.1 probe. This study was supported in part by the Physicians' Services Incorporation (PSI) Foundation grant 92-40. D.J.A.-H. is the recipient of a postdoctoral fellowship from the Ontario Ministry of Health.

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